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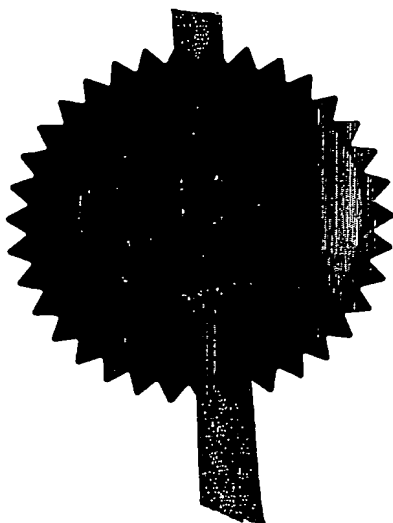
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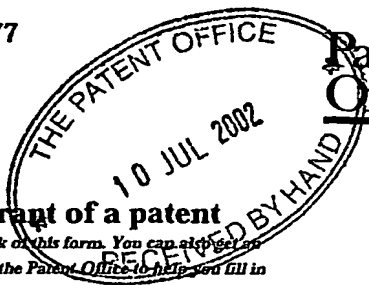
*Stephen Handley*

Dated 4 August 2003



Patents Form 1/77

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REP07135GB

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(The Patent Office will fill in this part)

0216026.5

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Cambridge Biotechnology Ltd.  
10 Wellington Street  
Cambridge  
CB1 1HW

Patents ADP number (if you know it)

8208761-001

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

4. Title of the invention

NUCLEIC ACID AMPLIFICATION METHOD

5. Name of your agent (if you have one)

Gill Jennings & Every

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Broadgate House  
7 Eldon Street  
London  
EC2M 7LH

Patents ADP number (if you know it)

745002

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Date of filing  
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Number of earlier application

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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

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- a) any applicant named in part 3 is not an inventor, or
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Description 6

Claim(s) 1

Abstract

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Priority documents

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Statement of inventorship and right to grant of a patent (*Patents Form 7/77*)

Request for preliminary examination and search (*Patents Form 9/77*)

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Any other documents  
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NO

11. For the applicant  
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

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Date

10 July 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

R E Perry

020 7377 1377

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## NUCLEIC ACID AMPLIFICATION METHOD

### Field of the Invention

This invention relates to a nucleic acid amplification method.

### Background of the Invention

5 WO01/06004 discloses a method for increasing the number of polynucleotides containing sequences corresponding to a mRNA species present in a sample. This method comprises 3 steps, i.e. reverse transcription of the mRNA species using a first heeled primer population, to provide first cDNA strands; synthesis of second cDNA strands from the first, using a second heeled primer population; and amplification of the first and second  
10 cDNA strands. The heel sequence of the primers used in the first and/or second steps contains a RNA polymerase promoter site. The primers used in the amplification comprise at least a portion of the first heel sequence and a portion of the second heel sequence. Either or each of the heel sequences preferably includes the nucleotide sequence of a restriction enzyme cleavage site; the polynucleotides that are produced may include  
15 concatomers, but can be treated with an agent that cleaves at this cleavage site, without affecting the desired amplicons.

### Summary of the Invention

The present invention is based on the discovery that the procedure disclosed in WO01/06004 can be simplified. According to the present invention, a method for  
20 increasing the number of polynucleotides containing sequences corresponding to a mRNA species present in a sample, comprises the steps of:

- (i) reverse transcription of the mRNA species using a heeled 5'-amplification primer (FAP) and a heeled 3'-amplification primer (TAP), wherein each primer sequence is unique and absent from the relevant genome, and each  
25 heel sequence includes a RNA polymerase promoter site, and the FAP includes a variable sequence, whereby the RNA is reverse-transcribed to produce double-stranded cDNA and then multiple cDNAs according to the variable sequence; and
- (ii) amplification of the cDNA using primers sufficiently complementary to  
30 FAP and TAP.

It will be evident that, in the present invention, reverse transcription and production of multiple cDNAs are conducted in one step. While the present invention

shares with the known procedure the use of a randomer sequence, so that the use of appropriate populations of varied FAP/TAP sequences gives multiple cDNAs, the novel procedure is simpler. The reverse transcription proceeds more efficiently, and fewer PCR cycles are required.

5 Further, by comparison with the known procedure, the need for rare restriction sites is avoided. The production of complex products is minimised, due in part to the use of unique sequences in FAP and TAP which are absent from the genome being investigated.

The present invention readily allows the inclusion of specific restriction sites, for manufacturing subtracted normalised and enriched cDNA libraries. It also allows the inclusion of specific restriction sites for lambda cloning, for the manufacture of single cell libraries. Again, specific restriction sites can be included, for fragmentation, for use as probes on the microarrays and filters. The procedure can readily be linked to a protocol for laser capture microdissection, e.g. as described by Fend *et al*, Am J Pathol.  
10 (1999)154(1):61-6.  
15

While bearing in mind the key distinctions between the present invention and the method disclosed in WO01/06004, the disclosure of the latter document is largely relevant, and is incorporated herein by reference.

An embodiment of the invention comprises a third step, i.e. (iii) transcription, to produce RNA run-offs from either end of the amplicons, e.g. using T3 RNA polymerase or T7 RNA polymerase. This is particularly suitable for use *in vitro* and when the number of cells is small.  
20

#### Description of Preferred Embodiments

The following description is of a procedure (abbreviated as "MEX") that illustrates the invention. As above, "FAP" and "TAP" are used as abbreviations for 5'- and 3'- amplification primers, respectively.  
25

##### **1) MEX RT reaction.**

In this step, specific priming sites for PCR amplification were engineered into appropriate ends of the cDNA population.

30 10µl of total RNA (prepared either by standard molecular biology protocols from cells/tissues/Laser Capture Microscopy cell harvests or from direct patch clamp harvesting) dissolved in water was added to 1µl of each of FAP-Randomer, i.e. ACT CCG

GGA ACC ATA GTG GCA CTC CTA ATT AAC CCT CAC TAA AGG GAG ATC GTAC (N)<sub>15</sub>, and TAP-RT, i.e. CAA GCA TTC AGT ATC TCG ACT GTA ATA CGA CTC ACT ATA GGG AGA GAT CGT AC (T)<sub>20</sub>, each at 10µM. This was heated to 70°C for 5 minutes before snap-cooling on ice for 2 minutes.

- 5        4µl of 5x Reverse Transcription buffer (Superscript II kit, Invitrogen), 2µl of 0.1M dithiothreitol (DTT, Superscript II kit, Invitrogen), 1µl of dNTPs (10mM mixture of each of dATP, dTTP, dGTP and dCTP), and 1µl of Superscript II reverse transcriptase enzyme (Superscript II at 200U/µl, Invitrogen) were added, and the reaction incubated at 42°C for 1 hour.

## 10    2) MEX amplification reaction

In this step, the reverse transcribed MEX-RT products were amplified for downstream molecular applications by PCR.

- 5µl of the RT reaction was amplified in a 100 µl PCR by the addition of the following reagents: 10µl Advantage II PCR buffer (Clontech), 1µl of dNTPs (10mM mixture of each of dATP, dTTP, dGTP and dCTP), 1µl FAP, i.e. ACT CCG GGA ACC  
15    ATA GTG GCA CTC C, at 10µM and 1µl TAP, i.e. CAA GCA TTC AGT ATC TCG ACT G, at 10µM and 77µl of sterile water.

Cycling parameters were as follows: 95°C for 1 minute, for one cycle, then up to 24 cycles of 95°C for 15 seconds, 65°C for 30 seconds, 68°C for 6 minutes.

## 20    MEX Mechanism of action

- In the first step, a 3'-heeled primer (TAP) initiates reverse transcription and first round cDNA synthesis through binding of a (T)<sub>20</sub> sequence, contained in the 3'-terminus of the primer, to polyA tails in the mRNA population. This primer contains a heel sequence for subsequent PCR amplification and a nested RNA polymerase promoter site  
25    (for T7 RNA polymerase) for subsequent *in vitro* transcription reactions. A second primer (FAP) is also included in the RT reaction; this initiates second round cDNA synthesis through semi-randomly priming from the first strand cDNA via a (N)<sub>15</sub> sequence incorporated at the 3'-terminus of the primer. This primer also contains a heel sequence for subsequent PCR amplification (NB This heel sequence is distinct from that in the 3'-  
30    primer) and a nested RNA polymerase promoter site (for T3 RNA polymerase) for

subsequent *in vitro* transcription reactions. The heel sequences are unique sequences as defined by their absence from the current genome databases.

In this way, a population of mRNAs is converted to double-stranded cDNAs that are anchored at their 3'-termini to the TAP and at their 5'-termini to the FAP.

5        The cDNA population therefore contains the following species of MEX fragments (where Gene X is any gene that has been reverse transcribed):

FAP SEQUENCE - T3 RNA POLYMERASE SEQUENCE  $-(N)_{15}$  - GENE X -  $(T)_{20}$  - T7  
RNA POLYMERASE - TAP SEQUENCE

#### **MEX from control RNAs**

10        Approx. 100ng of total brain RNA was reverse transcribed according to the MEX protocol described above. This was serially diluted and subjected to MEX amplification for either 9 cycles or 15 cycles. In addition, a control, where no amplification was performed, was also included. PCR was performed to determine the degree of amplification of actin mRNA transcripts in the cDNA and MEX amplified pools.

15        Actin transcripts could only be detected in unamplified cDNAs at a concentration of 50ng of total RNA equivalent. Transcripts could not be detected at cDNA concentrations below 5pg. In contrast, actin could be detected in the same cDNA samples at concentrations as low as 0.5pg after 9 cycles of MEX amplification and 50fg after 15 cycles of MEX amplification.

20        This experiment demonstrates that MEX amplification of cDNAs extends the range of detection beyond that where detection is possible by conventional RT PCR alone.

#### **MEX from Laser Capture Cells**

25        G-1 cultured cells were grown on glass microscope slides and processed for Laser Capture Microdissection (LCM). Defined numbers of cells were harvested, and RNA extracted using the Stratagene mico-RNA extraction kit. This RNA was reverse-transcribed using MEX RT primers and then subjected to MEX amplification for either 15 or 21 cycles. A proportion of the cDNA was also retained for analysis without MEX amplification. PCR was performed, using primers specific for actin, as the final assay of the efficiency of the MEX amplification.

30        Actin sequence was detected in as few as 50 cells after 15 cycles of MEX amplification and in as few as 10 cells after 21 cycles of MEX amplification. In contrast,

actin sequence was undetectable in samples that were not subject to MEX pre-amplification.

In conclusion, MEX can be used to link molecular techniques to LCM.

#### **MEX linked to *in vitro* transcription**

5        Approx. 100ng of total RNA was reverse-transcribed using MEX primers, serially diluted and subsequently MEX amplified for three, six or nine cycles. These products were *in vitro* transcribed (IVT) using the Megascript kit (Ambion) and reverse-transcribed for a second time using a conventional (dT)<sub>18</sub> primer and Superscripts II (Invitrogen). To gauge the relative efficiency of the amplifications, 30 cycles of gene specific PCR for the  
10    actin sequence were performed on these products. The combination of three cycles of MEX, when used in conjunction with IVT, was sufficient to enable the identification of actin transcripts in 5pg of total RNA. The application of an additional three cycles of MEX prior to IVT enabled this detection window to be extended to 50fg of total RNA. For comparison, 15 cycles of MEX were required to enable detection of actin in 50fg of  
15    RNA without the use of IVT.

In conclusion, MEX can be used in consort with IVT to extend the sensitivity of the detection, whilst minimizing cycle number. This may be important for increasing the amplicon yield from MEX whilst retaining linearity of representation in the amplicon pool. In addition, the production of RNA run-offs from MEX products allows the use of these  
20    products as probes on Affymetrix and other microarrays.

#### **MEX used in Target Identification**

To demonstrate the utility of MEX, c-fibres were harvested using LCM from an *in vitro* model of pain. This model was based on primary cultures of rat trigeminal ganglion cells treated with pro-nociceptive agents such as Nerve Growth Factor (NGF).  
25    RNA from these harvests was reverse-transcribed and amplified using 24 cycles of MEX. The resulting amplicon pool was then assayed with primers for genes with known association with nociception. When the amplicon pool was assayed prior to, and following, MEX amplification a distinct difference could be observed. Whilst control genes such as actin and cyclophilin (act and cyc) could be detected in the pre-MEX pool,  
30    other less abundant and biologically more important genes for the nociceptive response, i.e. TrkB receptor, PN3, sodium channel Beta 3 subunit, calcitonin gene related protein, vasointestinal peptide, neuropeptide Y, galanin and tyrosine hydroxylase (PN3,  $\beta$ 3, CGRP,



VIP, NPY, Gal, and TH) were absent. This was in contrast to the amplicon pool following MEX amplification, where the majority of these pro-nociceptive genes were readily detectable. This also validated the *in vitro* model of pain.

Therefore, MEX is of use in detecting genes of interest in small numbers of pathologically relevant cells. These genes may be used as targets for therapeutic intervention.

#### **MEX used in library construction**

The MEX primers have been designed to be compatible with many downstream applications. These include use in the manufacture of subtracted PCR libraries.

10 MEX products from small numbers of cells (100ng total RNA equivalent) were amplified from trigeminal ganglion primary cultures treated with or without nerve growth factor and purified to produce the starting material for the manufacture of subtracted libraries (top left hand panel). These were size-selected to remove small products and primer sequences (<50 base pairs). Linker molecules were ligated to these products to  
15 facilitate the subtraction (these were quality controlled in the middle top panel by using combinations of linker based / gene base PCR primers) and two rounds of suppression PCR subtraction applied to the MEX amplicons. This produced the final forward and reverse subtracted library pairs (shown in the top right hand panel). The libraries were quality controlled to ensure that "housekeeper" genes common to both subtraction pools  
20 had been removed during the subtraction process. This was demonstrated by the removal of actin transcripts from the subtracted libraries (bottom left hand panel). As a final check of the content of these libraries, products were cloned and sized (bottom right hand panel). A number of these clones were sequenced and differential expression was identified.

Therefore, MEX can be used to analyse gene expression in small numbers of cells  
25 and to identify genes that are differentially expressed between two or more cell types or cell types with distinct phenotypes.

CLAIMS

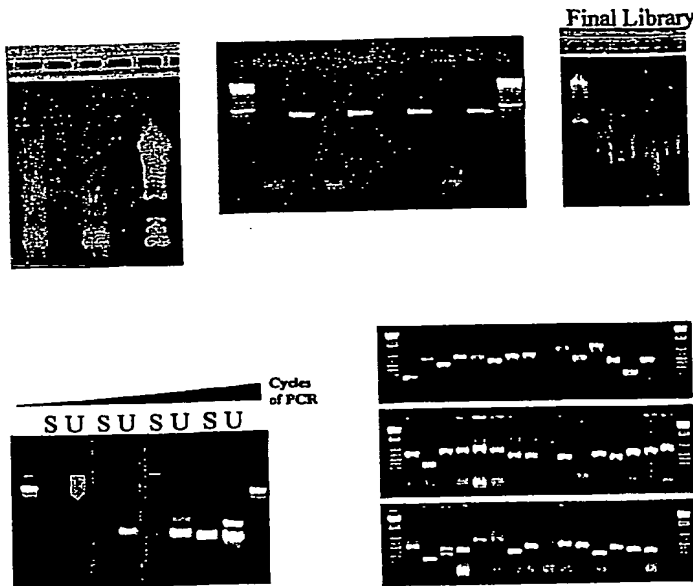
1. A method for increasing the number of polynucleotides containing sequences corresponding to a mRNA species present in a sample, the method comprising the steps of:

- 5 (i) reverse transcription of the mRNA species using a heeled 5'-amplification primer (FAP) and a heeled 3'-amplification primer (TAP), wherein each primer sequence is unique, and either or each heel sequence includes a RNA polymerase promoter site, and the FAP includes a variable sequence, whereby the RNA is reverse-transcribed to produce double-stranded cDNA and then multiple cDNAs according to the variable sequence; and
- 10 (ii) amplification of the cDNA using primers sufficiently complementary to FAP and TAP.

2. A method according to claim 1, which additionally comprises the step of:

- (iii) transcription, to produce RNA run-offs from either end of the amplicons.

## MEX Used in library construction



- MEX has been used to construct a PCR subtracted library, top panels, left raw amplicons, middle pre subtraction products and right final library).
- This library is enriched for key marker genes (bottom left panel) and contains clones that are known to be differentially expressed as a consequence of the treatment

MEX has been used to make a PCR subtracted library

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 03/02989

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C12Q1/68 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 06004 A (UNIV CAMBRIDGE TECH; COX PETER (GB); RICHARDSON PETER (GB)) 25 January 2001 (2001-01-25) cited in the application the whole document	1-15,17, 18
A	WO 00 08208 A (MEDICAL RES COUNCIL; RICHARDSON PETER JOHN (GB); DIXON ALISTAIR K) 17 February 2000 (2000-02-17)	
P,X	EP 1 275 738 A (HOFFMANN LA ROCHE ; ROCHE DIAGNOSTICS GMBH (DE)) 15 January 2003 (2003-01-15) the whole document, in particular figure 1	1,2,6, 14,15

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Date of the actual completion of the international search

7 October 2003

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/68 03/02989

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0106004	A	25-01-2001	AU 6695600 A	05-02-2001
			CA 2378070 A1	25-01-2001
			WO 0106004 A2	25-01-2001
			EP 1196639 A2	17-04-2002
			JP 2003505034 T	12-02-2003
WO 0008208	A	17-02-2000	AU 5294399 A	28-02-2000
			WO 0008208 A2	17-02-2000
EP 1275738	A	15-01-2003	EP 1275734 A1	15-01-2003
			EP 1275738 A1	15-01-2003
			JP 2003093058 A	02-04-2003
			US 2003113754 A1	19-06-2003

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 03/02989

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C12Q1/68 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 06004 A (UNIV CAMBRIDGE TECH; COX PETER (GB); RICHARDSON PETER (GB)) 25 January 2001 (2001-01-25) cited in the application the whole document	1-15, 17, 18
A	WO 00 08208 A (MEDICAL RES COUNCIL; RICHARDSON PETER JOHN (GB); DIXON ALISTAIR K) 17 February 2000 (2000-02-17)	
P, X	EP 1 275 738 A (HOFFMANN LA ROCHE ; ROCHE DIAGNOSTICS GMBH (DE)) 15 January 2003 (2003-01-15) the whole document, in particular figure 1	1, 2, 6, 14, 15

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7 October 2003

Date of mailing of the international search report

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Name and mailing address of the ISA

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Pinta, V

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/68 03/02989

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0106004	A	25-01-2001	AU 6695600 A	05-02-2001
			CA 2378070 A1	25-01-2001
			WO 0106004 A2	25-01-2001
			EP 1196639 A2	17-04-2002
			JP 2003505034 T	12-02-2003
WO 0008208	A	17-02-2000	AU 5294399 A	28-02-2000
			WO 0008208 A2	17-02-2000
EP 1275738	A	15-01-2003	EP 1275734 A1	15-01-2003
			EP 1275738 A1	15-01-2003
			JP 2003093058 A	02-04-2003
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